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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

A METHOD FOR TRANSPLANTING LYMPHOHEMATOPOIETIC CELLS INTO A MAMMAL

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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☐ Yes, the name of the U.S. Government agency and the Government contract number are:

☐ Applicant claims small entity status under 37 C.F.R. § 1.27.

Respectfully submitted,

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PROVISIONAL APPLICATION

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TITLE : A METHOD FOR TRANSPLANTING LYMPHOHEMATOPOIETIC CELLS INTO A MAMMAL

A METHOD FOR TRANSPLANTING LYMPHOHEMATOPOIETIC CELLS INTO A MAMMAL

Abstract

Background In vivo expansion of gene-modified cells would be a promising approach in the field of hematopoietic stem cell gene therapy. To this end, we previously developed selective amplifier gene (SAG), a chimeric gene encoding the granulocyte colony-stimulating factor (G-CSF) receptor (GCR) as a growth-signal generator and the hormone-binding domain of the steroid receptor as a molecular switch. We have already reported that hematopoietic cells retrovirally transduced with the SAG can be expanded in a steroid-dependent manner in vitro and in vivo in mice and nonhuman primates. In this study, we have developed a new generation SAG, in which the erythropoietin (EPO) receptor (EPOR) is utilized instead of the steroid receptor as a molecular switch. **Methods** Two EPO-driven SAGs were constructed; EPORGCR and EPORMpl, containing the GCR and c-Mpl as a signal generator, respectively. First, to compare the steroid-driven and EPO-driven SAGs, Ba/F3 cells were transduced with these SAGs. Next, to examine whether GCR or c-Mpl is the more suitable signal generator of the EPO-driven SAG, human cord blood $CD34^+$ cells were transduced with the two EPO-driven SAGs (EPORMpl and EPOGCR). Finally, we examined the in vivo efficacy of EPORMpl in mice. Irradiated mice were transplanted with EPORMpl-transduced bone marrow cells followed by administration of EPO.

Results The EPO-driven SAGs were shown to induce more rapid and potent proliferation of Ba/F3 cells than the steroid-driven SAGs. The EPORMpl induced more efficient EPO-dependent proliferation of the human cord blood $CD34^+$ cells than the EPORGCR in terms of total $CD34^+$ cell, c-Kit^+ cell, and clonogenic progenitor cell

(CFU-C) numbers. In the transplanted mice the transduced peripheral blood cells significantly increased in response to EPO.

Conclusion The new generation SAGs, especially EPORMpl, are able to efficiently confer an EPO-dependent growth advantage on transduced hematopoietic cells in vitro and in vivo in mice.

Key Words: hematopoietic stem cells, gene therapy, $CD34^+$ cells, selective amplifier gene, in vivo expansion, retroviral vector

Introduction

One of the major obstacles associated with hematopoietic stem cell (HSC) gene therapy is the low efficiency of gene transfer into human HSCs with retroviral vectors [1]. The ability to positively select cells containing potentially therapeutic genes in vivo would represent an important tool for the clinical application of HSC gene therapy. One strategy of in vivo selection of transduced hematopoietic cells is to utilize drug-resistance genes such as the multidrug resistance 1 (MDR-1) gene [2], mutant dihydrofolate reductase (DHFR) gene [3], or DNA alkyltransferase gene [4-6]. Although the strategy has been successful in mice, it has proven less effective thus far in human subjects or nonhuman primates [7-9], and administration of agents such as taxol (for MDR-1 selection) or methotrexate (for DHFR selection) is highly toxic.

Another strategy of in vivo positive selection of transduced cells is to confer a direct proliferation advantage on gene-modified cells relative to their untransduced counterparts. We developed a chimeric gene designated "selective amplifier gene" (SAG) which encodes a chimeric receptor between the granulocyte colony-stimulating factor (G-CSF) receptor (GCR) and the hormone-binding domain of the estrogen or tamoxifen receptor. The GCR moiety is a growth signal generator and the estrogen receptor (ER) moiety is a molecular switch to regulate (turn on or off) the growth signal generated from the GCR. We previously showed that hematopoietic cells transduced with the SAG can be selectively expanded in an estrogen- or tamoxifen-dependent manner in vitro [10-13] and in vivo in mice and nonhuman primates [14,15]. In

nonhuman primates, however, some animals that received the SAG did not show an increase in transduced cells in response to estrogen or tamoxifen, suggesting that the SAG was not potent enough to achieve in vivo expansion in all monkeys [15].

The utilization of the steroid receptor as a molecular switch may have attenuated the potency of the SAG. The estrogen-mediated dimerization of the chimeric molecule may be less efficient than the natural ligand (G-CSF)-mediated dimerization. In fact, the fusion protein between the GCR and estrogen receptor responds to G-CSF more efficiently than to estrogen [10]. To rectify this problem, we utilized the erythropoietin receptor (EPOR) instead of the steroid receptor as a molecular switch. Since the EPOR is a member of the cytokine receptor superfamily [16], the fusion proteins between the EPOR and other cytokine receptors such as the GCR should be more stable and compatible than the prototype fusion protein. In addition, the EPOR is not expressed on immature hematopoietic cells and thus can be used as a selective switch for these cells [17]. Of note, recombinant human erythropoietin (EPO) has already been used widely in clinical application and can be administered repeatedly to human subjects without serious adverse effects [18,19].

On the other hand, as a growth signal generator, we tried to use the thrombopoietin (TPO) receptor, c-Mpl, in addition to the GCR. It has been reported that c-Mpl is expressed on very immature hematopoietic cells and that TPO actually stimulates the growth of these cells [20-23]. In fact, the cytoplasmic fragment of c-Mpl has been already used for cell expansion [24,13]. The intracellular signal from c-Mpl may thus be more appropriate than that from the GCR for expansion of hematopoietic

stem/progenitor cells. In the present study, we examined the efficacy of these new generation SAGs in vitro and in vivo in mice.

Materials and methods

Cell lines

Ba/F3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), and 1 ng/ml recombinant mouse IL-3 (rmIL-3; Gibco-BRL). The ecotropic packaging cell line BOSC23 [25] and human embryonic kidney 293T cells were maintained in DMEM containing 10% FBS (Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL).

Plasmid construction

The wild-type human erythropoietin receptor (EPORwt) cDNA was obtained from pCEP4-EPOR (kindly provided by Dr. R. Kralovics, University of Alabama, UK) [26]. The fragment containing the murine phosphoglycerate kinase (pgk) promoter and neomycin phosphotransferase gene (neo) (EcoRI-BamHI) in the retroviral plasmid pMSCV2.2 (kindly provided by Dr. R. G. Hawley, University of Toronto, Canada) [27] was replaced by the EPORwt cDNA (EcoRI-BamHI) to construct pMSCV-EPORwt.

pMSCV-EPORGCR and pMSCV-EPORMpl were constructed as follows. The cytoplasmic region of murine G-CSF receptor (GCR) cDNA was obtained by PCR using pMSCV-AY703FGCRER as a template [11] with the primer pair 5'-AAG GAT

CCA AAC GCA GAG GAA AGA AGA CT-3' and 5'-AAG TCG ACC TAG AAA CCC CCT TGT TC-3'. The cDNA coding to the cytoplasmic region of human TPO receptor (c-Mpl) was obtained by PCR using pcDNA3.1-c-Mpl (provided by Dr. M. Takatoku, Jichi Medical School, Tochigi, Japan) [28] as a template with the primer pair 5'-AAG GAT CCA GGT GGC AGT TTC CTG CA-3' and 5'-CGG TCG ACT CAA GGC TGC TGC CAA TA-3'). The fragment containing the extracellular plus transmembrane region of the human EPOR cDNA was obtained by PCR using pCEP4-EPOR as a template with the primer pair 5'-CTC GGC CGG CAA CGG CGC AGG GA-3' and 5'-AAG GAT CCC AGC AGC GCG AGC ACG GT-3'. The fragment containing the extracellular plus transmembrane region of human EPOR cDNA and the fragment containing the cytoplasmic region of murine GCR or human c-Mpl was cloned into the EcoRI-SalI site of pBluescript SK (pSK; Stratagene, La Jolla, CA) to construct pSK-EPOGCR or pSK-EPOMpl, respectively. The pgk promoter/neo cassette (EcoR-SalI) in pMSCV was replaced by the EcoRI-SalI fragment containing the EPORGCR or EPORMpl cDNA each from pSK-EPORGCR or pSK-EPORMpl, respectively. The resultant construct was designated as pMSCV-EPORGCR or pMSCV-EPORMpl, respectively.

pMSCV-EPORwt-ires-mitoEYFP, pMSCV-EPORGCR-ires-mitoEYFP, and pMSCV-EPORMpl-ires-mitoEYFP were constructed as follows. The internal ribosome entry site (ires) sequence derived from pIRES-EGFP (Clontech, Palo Alto, CA) and the mitoEYFP cDNA derived from pEYFP-Mito (Clontech) were inserted into the PstI-BamHI site and the SpeI-NotI site of pSK, respectively. The resultant plasmid was

pSK-ires-mitoEYFP. The mitEYFP cDNA encodes the enhanced yellow fluorescent protein (enhanced YFP, EYFP) linked to a mitochondria localization signal sequence so that EYFP is sequestered inside the mitochondria, thus circumventing the presumed toxicity of YFP [29]. The blunted fragment encoding the ires-mitoEYFP cDNA was ligated into the ClaI blunted site of pMSCV-EPORwt, pMSCV-EPORGCR, and pMSCV-EPORMpl to obtain pMSCV-EPORwt-ires-mitoEYFP, pMSCV-EPORGCR-ires-mitoEYFP, and pMSCV-EPORMpl-ires-mitoEYFP, respectively. The final plasmids were certified as correct by sequence analysis.

Retroviral vectors

To obtain ecotropic retroviral vectors, BOSC23 cells were transfected with mouse stem cell virus (MSCV)-based retroviral plasmids (derivatives from pMSCV, see above) using Lipofectamine Plus (Invitrogen, San Diego, CA) according to the manufacturer's protocol and the supernatants containing the ecotropic retroviral vectors were harvested 48-72 hours after transfection. The titers were $1x10^6$/ml as assessed by RNA dot-blot. To obtain amphotropic retroviral vectors, 293T cells were transfected with MSCV-based retroviral plasmids along with pCL-Ampho (Imugenex, San Diego, CA) using Lipofectamine Plus (Invitrogen) and the supernatants containing the amphotropic retroviral vectors were harvested 48-72 hours after transfection. The titers were $1x10^6$/ml as assessed by RNA dot-blot.

Retroviral transduction and culture

Ba/F3 cells were suspended in 1 ml retroviral supernatant containing 10 ng/ml rmIL-3 at a density of $1x10^5$ cells/ml, and transferred to 12-well plates coated with 20 $\mu g/cm^2$ of RetroNectin (Takara Bio, Shiga, Japan) [30]. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours. During this period, culture medium was replaced by fresh viral supernatant twice (every 12 hours). After retroviral infection, YFP-positive cells were isolated using an EPICS ELITE cell sorter (Coulter, Miami, FL) according to the manufacturer's instructions. The purity of sorted YFP-positive cells was greater than 98%. The sorted Ba/F3 cells were subjected to further liquid culture (described above) or cell proliferation assays (see below).

Human cord blood $CD34^+$ cells (BioWhittaker, Walkersville, MD) were thawed and placed in 12-well plates coated with 20 $\mu g/cm^2$ of RetroNectin (Takara Bio) and cultured for 24 hours at 37°C with 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL) supplemented with 10% FBS (Hyclone, Logan, UT), 50 ng/ml recombinant human interleukin 6 (rhIL-6; Ajinomoto, Osaka, Japan), 100 ng/ml recombinant human stem cell factor (rhSCF; Amgen, Thousand Oaks, CA), 100 ng/ml recombinant human Flt-3 ligand (Research Diagnostic, Flanders, NJ), and 100 ng/ml recombinant human thrombopoietin (rhTPO; Kirin, Tokyo, Japan). The cells were then resuspended in 1 ml viral supernatant containing the same cytokines as described above at a starting density of $1x10^5$ cells/ml. During the transduction period (48 hours), culture medium was replaced by fresh viral supernatant 4 times (every 12 hours). After

retroviral transduction, human cord blood $CD34^+$ cells were washed twice and cultured in IMDM medium containing 10% FBS (Hyclone) and 1% penicillin/streptomycin in the presence of 10 ng/ml EPO in a 37°C 5% CO_2 incubator. The cells were subjected to flow cytometry or colony assay (see below) on the indicated days.

Cell proliferation assay

Ba/F3 proliferation assay was performed using the CellTier 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. In brief, 20 µl MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium)-labeling mixture was added to each well of 96-well dishes containing cells to be assayed. Following incubation at 37°C for 2 hours, the spectrophotometric absorbance was measured at the wavelength of 490 nm and 650 nm. A_{490}-A_{650} values were used to determine Ba/F3 cell proliferation. Experiments were conducted in triplicate.

Flow cytometry

Human cord blood $CD34^+$ cells were washed and resuspended in CellWASH (Becton Dickinson, San Jose, CA). The cells were then incubated with phycoerythrin (PE)-labeled anti-c-Kit (Nichirei, Tokyo, Japan), PE-labeled anti-glycophorin A (Nichirei), PE-labeled anti-CD41 (Nichirei), or PE-labeled anti-CD15 (Immunotech,

Marseille, France) at 4°C for 30 minutes. The cells were washed once and subjected to a FACSCalibur (Becton Dickinson) using excitation at 488 nm. Untransduced cells served as negative controls.

For mouse blood samples, blood cells were suspended in ACK lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA; Wako, Osaka, Japan) to dissolve red blood cells. The cells were washed once and subjected to a FACSCalibur (Becton Dickinson) using excitation at 488 nm.

Colony assay and PCR

Human cord blood $CD34^+$ cells were plated in 35-mm dishes with α-minimum essential medium (Gibco-BRL) containing 1.2% methylcellulose (Shinetsu Kagaku, Tokyo, Japan) supplemented with 20% FBS (Intergen, Purchase, NY) and 1% bovine serum albumin (Sigma, St. Louis, MO) in the presence of 100 ng/ml rhSCF, 100 ng/ml rhIL-6, and 100 ng/ml recombinant human interleukin 3 (rhIL-3; PeproTech, London, UK), or in the presence of 20 ng/ml of recombinant human erythropoietin (rhEPO) alone. After incubation for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies were scored under an inverted microscope. The experiments were performed in triplicate.

Colonies in methylcellulose culture were picked up under an inverted microscope, suspended in 50 μl of distilled water, and digested with 20 μg/ml proteinase K (Takara) at 55°C for 1 hour followed by incubation at 99°C for 10 minutes. PCR was

performed to amplify the 351-bp sequence using the EYFP sense primer (5'-CGT CCA GGA GCG CAC CAT CTT C-3') and antisense primer (5'-AGT CCG CCC TGA GCA AAG ACC-3'). To certify the initial DNA amounts, the β-actin genomic DNA fragment was simultaneously amplified using the sense primer (5'-CAT TGT CAT GGA CTC TGG CGA CGG-3') and antisense primer (5'-CAT CTC CTG CTC GAA GTC TAG GGC-3'). Amplification conditions were 95°C for 1minute, 55°C for 30 seconds, and 72°C for 30 seconds with 35 cycles.

Mouse transplantation

Eight-week-old C57Bl/6 mice (Charles River Japan, Yokohama, Japan) intraperitoneally received 150 μg/kg 5-fluorouracil (Sigma). Forty-eight hours after injection, bone marrow cells were harvested from the femora of each mouse. Cells were cultured in IMDM (Gibco-BRL) containing 20% FBS (Hyclone) and 20 ng/ml rhIL-6 and 100 ng/ml recombinant rat SCF (provided by Amgen) for 48 hours. The cells were then placed in 6-well plates coated with 20 $\mu g/cm^2$ of RetroNectin (Takara Bio) and resuspended in IMDM (Gibco-BRL) supplemented with 10% FBS (Hyclone) and the aforementioned cytokines at a starting density of 5×10^5 cells/ml. During the transduction period (48 hours), culture medium was replaced by fresh viral supernatant 4 times (every 12 hours). The cells were harvested after a total of 96 hours (4 days) in culture, washed with phosphate-buffered saline (PBS) 3 times, and injected into 8-week-old female C57/B16 mice that had been irradiated with 800 cGy. After

transplantation, some mice received recombinant mouse EPO (rmEPO; 200 IU/kg Roche Diagnostics) in a total volume of 100 µl via the tail vein three times a week. To avoid development of anemia after drawing blood from the transplanted mice, blood was transfused into the mice via the tail vein at the time of blood drawing. The blood for transfusion was drawn from donor C57/Bl6 mice and pooled. It was irradiated with 20 Gy and diluted with physiological salt solution prior to transfusion. Peripheral blood mononuclear cells of the recipient mice were analyzed for EYFP expression by flow cytometry.

Results

A new generation SAG

The structure of SAGs is shown in Figure 1. The prototype SAG (steroid-driven SAG) is a chimeric gene encoding the G-CSF receptor (GCR) and the estrogen receptor hormone-binding domain. In the GCR, the ligand (G-CSF)-binding domain was deleted to remove the responsiveness to endogenous G-CSF [10]. The tyrosine residue at the 703rd amino acid in the GCR was replaced by phenylalanine to hamper the differentiation signal [11]. In addition, another mutation (G525R) was introduced in the estrogen receptor hormone-binding domain to evade the responsiveness to endogenous estrogen without impairing the responsiveness to synthetic hormones such as tamoxifen [12]. In this study, we constructed a new generation SAG, in which the erythropoietin

(EPO) receptor (EPOR) is utilized instead of the estrogen or tamoxifen receptor as a molecular switch. Two types of EPO-driven SAG were constructed, EPORGCR and EPORMpl, containing the GCR gene and the thrombopoietin (TPO) receptor (c-Mpl) gene, respectively, as a growth-signal generator.

In vitro effects of the EPO-driven SAG on Ba/F3

Bicistronic retroviral vectors were generated which express the EPO-driven SAG or wild-type EPOR (EPORwt) gene as the first cistron and the EYFP gene as the second cistron. The vectors were infected into Ba/F3 cells. Ba/F3 is a mouse pro-B cell line and the cells require IL-3 for growth. YFP-positive cells were isolated (>98% purity) and stimulated by EPO at various concentrations (Figure 2A). All the cells acquired the ability of EPO-dependent growth and were able to proliferate even in the absence of IL-3. Ba/F3 cells expressing either EPORwt, EPORGCR, or EPORMpl reached the maximum growth levels by adding 1-100 ng/ml EPO (Figure 2A). Endogenous EPO will not induce a significant proliferative response of the cells, since the physiological range of serum EPO concentrations is below 0.1 ng/ml.

We compared the EPO- and steroid-driven SAGs in terms of their ability to expand Ba/F3 cells. The Ba/F3 cells expressing the EPO-driven SAGs were cultured in the presence of 10 ng/ml EPO and the Ba/F3 cells expressing the steroid-driven SAG were cultured in the presence of 10^{-7} M tamoxifen [12]. The Ba/F3 cells expressing either of the two EPO-driven SAGs proliferated in the presence of EPO to the same

extent as the parental Ba/F3 cells in the presence of IL-3. Of note, the EPO-driven SAG (EPORGCR) expanded Ba/F3 cells by around 10^4-fold more than the steroid-driven counterpart (ΔGCRTmR) after 2 weeks of culture (Figure 2B), indicating that the molecular switch using the EPOR is more efficient than that using the tamoxifen receptor despite the inclusion of the same signal generator (GCR) in the SAGs. Thus, we used EPO-driven SAGs for subsequent experiments.

In vitro effects of the EPO-driven SAGs on human CD34$^+$ cells.

To examine whether GCR or c-Mpl is the more suitable signal generator of the EPO-driven SAG, human cord blood CD34$^+$ cells were used as targets. CD34$^+$ cells were transduced with bicistronic retroviral vectors which express the EPO-driven SAG as the first cistron and the EYFP gene as the second cistron. After transduction, 27.3 ± 4.7% of the cells fluoresced (YFP-positive). The transduced CD34$^+$ cells were then cultured in liquid medium in the presence of EPO. The fraction of YFP-positive cells increased over time, and virtually all (>95%) of the cells became YFP-positive during a 2-week culture with EPO. This suggests that the EPO-driven SAGs are able to confer a growth advantage on human CD34$^+$ cells. As shown in Figure 3, although the cells transduced with EPORwt proliferated most quickly, the cell number already began to decrease within 2 weeks after the culture initiation. The cells transduced with EPORGCR grew slowly compared with the others, but began to decrease in number by week 3. On the other hand, the cells transduced with EPORMpl proliferated the

longest (1 month) in the presence of EPO and the cell number increased by 10^4-fold over this period.

Characterization of the c-Mpl signal of SAG.

The transduced $CD34^+$ cells were then examined for the expression of c-Kit, a primitive hematopoietic cell marker, by flow cytometry (Figure 4). The $c\text{-}Kit^+$ fraction decreased over time, implying that the cells differentiated during culture. The $c\text{-}Kit^+$ fraction in the cells transduced with EPORMpl, however, was relatively high (33%) at week 3 in liquid culture, whereas the $c\text{-}Kit^+$ fraction decreased to 10% or lower in the cells transduced with EPORwt or EPORGCR at the same time point. These results demonstrate that the c-Mpl signal preserved more $c\text{-}Kit^+$ immature hematopoietic cells than the other signals.

To examine the EPO-driven SAGs for their ability to expand hematopoietic progenitor cells, $CD34^+$ cells transduced with the EPO-driven SAGs were cultured in semisolid (methylcellulose) media in the presence of multiple cytokines (IL-3, IL-6 and SCF) or EPO alone. Table 1 summarizes the results. The cells transduced with the EPO-driven SAGs formed many colonies in the presence of EPO and almost all of them (94 to 100%) contained the provirus as assessed by individual colony PCR. In contrast, 25-38% of the colonies formed by cells in the presence of multiple cytokines contained the provirus. This result shows that the EPO-driven SAGs are able to confer an EPO-dependent growth advantage at the level of clonogenic progenitor cells. The cells transduced with the EPO-driven SAGs before (day 0) and after (day 7) liquid culture

with EPO were placed in semisolid media in the presence of EPO without other cytokines, and the resultant myeloid and erythroid colonies were counted. As shown in Figure 5, during the liquid culture with EPO, the transduction by EPORMpl resulted in the highest levels of clonogenic progenitor cell expansion by more than 10-fold.

We then examined whether cells transduced with the EPO-driven SAGs would show any specific lineage preference after liquid culture with EPO. The transduced $CD34^+$ cells were cultured in liquid medium containing EPO. During the culture, the expression of various differentiation markers was examined by flow cytometry (Figure 6). As expected, the erythroid marker (glycophorin A) was expressed in almost all (93%) cells transduced with EPORwt at day 14. The myeloid marker (CD15) was expressed in 24% of cells transduced with EPORGCR at day 7 (data not shown), but fell to 1% by day 14. Thus, EPORGCR induced very few cells to differentiate toward the myeloid lineage despite the inclusion of the GCR moiety as a signal generator. One reason may be that a point mutation (Y703F) was introduced into the GCR cDNA to attenuate the granulocytic differentiation signal (Figure 1) [11]. On the other hand, cells transduced with EPORMpl expressed all of these markers at relatively high levels at day 14; the megakaryocytic marker (CD41) (46%), glycophorin A (58%) and CD15 (11%). Thus, the cells expanded by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. We therefore decided to utilize EPORMpl as an SAG for subsequent in vivo experiments in mice.

In vivo expansion of gene-modified cells

Finally, we examined the efficacy of the EPOMpl-type SAG in vivo in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the MSCV-based vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice and, after hematopoietic reconstitution, YFP expression was examined in the peripheral blood by flow cytometry to see whether the EPOMpl-transduced cells would increase in response to EPO administration. In mice, however, even drawing a small volume of blood will result in the elevation of endogenous EPO concentrations [31,32]. We also confirmed that sequential blood-drawing caused an elevation of endogenous serum EPO concentrations in mice (data not shown). Therefore, drawing blood from the transplanted mice may result in the expansion of transduced hematopoietic cells. To avoid development of anemia due to blood drawing, we transfused mice at the time of blood drawing. As a result, the mice did not develop anemia, and thus the elevation of endogenous EPO concentration was prevented.

In the group receiving EPORMpl, YFP-positive cells increased in response to the EPO administration (n=6), although YFP-positive cells remained unchanged without EPO administration (n=4) (Figure 7A). On the other hand, in the control group (n=6) receiving YFP alone without EPORMpl, YFP-positive cells remained unchanged at around 10% in the peripheral blood regardless of EPO administration (Figure 7B). In the mice receiving EPORMpl, a significant increase (paired *t*-test, $p<0.05$) in

YFP-positive cells was observed 4 weeks after the initiation of EPO administration (Figure 7A). The increase was attributable to that in granulocytes and monocytes (data not shown). However, the increase seemed transient, as a significant increase was no longer observed at further time points.

Discussion

Although a few HSC gene therapy trials have proven successful [33,34], most attempts have been hampered by the low efficiency of gene transfer into HSCs. To overcome the problem, we have previously developed a method of selective in vivo amplification of transduced hematopoietic cells using a "selective amplifier gene" (SAG) which encodes a fusion protein consisting of a growth signal generator and its molecular switch. The prototype SAG encodes a fusion protein between the GCR and the estrogen or tamoxifen receptor, and confers a growth advantage on gene-modified hematopoietic cells in an estrogen- or tamoxifen-inducible fashion in vivo [15,16]. In the present study, we developed a new generation SAG which utilizes the EPOR as a molecular switch instead of the steroid receptor. The EPO-driven SAG encodes a fusion protein between the extracellular plus transmembrane domain of the EPOR and the cytoplasmic domain of the GCR or c-Mpl. The results reported here indicated that the SAG utilizing the EPOR as a molecular switch is more efficient for hematopoietic cell proliferation than that utilizing the steroid (or tamoxifen) receptor despite the inclusion of the same signal generator in the SAGs.

Cytokine receptors generate the growth signal through ligand-induced dimerization. Unliganded cytokine receptor dimers, however, exist in a conformation that prevents signal generation but then undergo a ligand-induced conformation change that allows signal generation [35,36]. Thus, dimerization is necessary but not sufficient for optimal signal generation. The EPO-driven SAG might have allowed more effective

ligand-induced conformation change than the steroid-driven SAG. Similar to our chimeric receptors, Blau et al. developed a cell growth switch that is a cytokine receptor-FK506 binding protein (FKBP) fusion gene to confer inducible proliferation to transduced cells [37,38]. In their system, cytokine receptor signal is turned on by treatment with a synthetic dimerizer FK1012 or its derivatives. However, it remains unclear whether their chimeric protein would allow effective ligand-induced conformation change to the same extent as the EPO-driven SAG.

We also showed that the c-Mpl signal expanded clonogenic progenitor cells (CFU) far more efficiently than the EPOR or GCR signal. In addition, the cells expanded by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. Other investigators have also shown that the c-Mpl signal is able to efficiently support the growth of transduced murine bone marrow cells [39]. Taken together, the intracellular signal from c-Mpl may be suitable for reliable expansion of immature hematopoietic cells.

We have demonstrated that EPORMpl can confer an EPO-dependent growth advantage on the transduced hematopoietic cells in vivo in a mouse transplantation model. It should be noted that EPORMpl contains the human c-Mpl and may not have worked well in mouse cells. It would be more predictive to examine the efficacy of the EPORMpl in nonhuman primates. We will evaluate the efficacy of EPOMpl-type SAG in the setting of a nonhuman primate transplantation protocol. In mice, the increase of transduced cells with EPORMpl seemed transient, as was the case with chimeric genes reported by other investigators [40,41]. The method may not result in the selection of

transduced cells at the HSC level. The long terminal repeat (LTR) promoter may not express the transgene in HSCs. Alternatively, the c-Mpl signal may not induce proliferation of HSCs. Thus, the selection of transduced cells may occur only within the differentiated progeny of transduced HSCs, not at the level of transduced HSCs themselves. In order to obtain clinically relevant effects, repeated EPO administration would be required. In such cases polycythemia may be induced, however we believe that EPO is much safer than steroid which is an inducer of the earlier SAGs. Polycytemia induced by EPO can be treated by phlebotomy easily and safely, but side effect induced by steroid may not be well treated or controlled.

With the EPO-driven SAG, therapeutic effects may result from continuously elevated levels of endogenous EPO in patients with chronic anemia such as thalassemia. When anemia is ameliorated and endogenous EPO levels return to physiological levels, the positive selection system is then "automatically" turned off. This "leave it to patients" system would be convenient. However, a safety concern may be raised regarding leukemogenesis, as the SAG proliferation signal is persistently turned on in vivo by endogenous EPO, although physiological levels of EPO will not induce a significant proliferative response. Since a set of EPO-mimetic peptides or a modified EPO such as the erythropoiesis stimulating protein (NESP) has been developed [42,43], it may be possible to develop an EPO-driven SAG containing a mutant EPOR which does not bind to endogenous EPO but binds to the EPO-mimetic peptides or modified EPO.

Figure Legends

Figure 1. The structure of SAGs. The GCRER is a prototype of the selective amplifier gene (SAG); a chimeric gene encoding the G-CSF receptor (GCR) as a growth-signal generator and the estrogen receptor hormone-binding domain (ER-HBD) as a molecular switch. In ΔY703F-GCRTmR, the G-CSF-binding domain was deleted from the GCR gene to abolish responsiveness to endogenous G-CSF, a point mutation (Y703F) was introduced to the GCR moiety to disrupt the differentiation signal generated by the GCR, and another point mutation (G525R) was introduced to the ER-HBD moiety to evade responsiveness to endogenous estrogen without impairing responsiveness to a synthetic hormone tamoxifen. In the new SAG, the erythropoietin (EPO) receptor (EPOR) was utilized instead of the estrogen or tamoxifen receptor as a molecular switch. To construct it, the intracellular domain of the wild-type EPOR (EPORwt) gene was replaced by that of the GCR or thrombopoietin receptor (c-Mpl) gene as a growth-signal generator.

Figure 2. The EPO-driven SAG efficiently stimulates Ba/F3 cell growth. (A) EPO-dependent growth of Ba/F3 cells by introduction of the EPO-driven SAG. BaF3 cells were transduced with the EPORwt (closed triangle), EPORGCR (closed square) or EPORMpl gene (closed circle) each along with the EYFP gene by bicistronic retroviral vectors. YFP-positive cells were sorted (>98%) and treated with EPO at various concentrations. The proliferation assay (see Materials and Methods) was performed on

day 0 and day 2, and the ratio of day 2 A_{490}-A_{650} to day 0 A_{490}-A_{650} (means±SD of triplicate) is shown. An arrow indicates the physiological range of EPO concentrations in human plasma. (B) The EPO-driven SAG triggers higher levels of cell proliferation than the steroid-driven SAG. The parental Ba/F3 cells (open diamond) were cultured in the presence of IL-3 (10 ng/ml). Ba/F3 cells transduced with the EPORwt (closed triangle), EPORGCR (closed square), or EPORMpl gene (closed circle) were cultured in the presence of EPO (10 ng/ml). Ba/F3 cells transduced with the ΔGCRTmR gene (open triangle) were cultured in the presence of tamoxifen (10^{-7} M). Accumulative cell numbers calculated with means of triplicate are shown in log scale.

Figure 3. The EPORMpl is the most potent amplifier for human cord blood $CD34^+$ cells. Human cord blood $CD34^+$ cells were transduced with the EPOwt (closed triangle), EPORGCR (closed square), or EPORMpl gene (closed circle) each along with the EYFP gene by bicistronic retroviral vectors. Untransduced cells are also shown (open diamond). The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. Virtually all the cells (>95%) became YFP-positive by week 2. Accumulative cell numbers calculated with the means of triplicate are shown in log scale.

Figure 4. The EPOR-Mpl preserves c-Kit^+ cells most efficiently. Human cord blood $CD34^+$ cells were transduced with the EPOwt (black), EPORGCR (gray), or EPORMpl gene (white) by the same retroviral vectors in Figure 3. The cells were then

cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. On the indicated days, aliquots of the cells were examined for c-Kit expression by flow cytometry. The percentages of cKit-positive cells are shown.

Figure 5. The EPOR-Mpl expands clonogenic progenitor cells most efficiently. Human cord blood $CD34^+$ cells were transduced with the EPOwt, EPORGCR or EPORMpl gene by the same retroviral vectors in Figure 3. The untransduced and transduced cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO for 7 days. The cells before (day 0) and after (day 7) the liquid culture were plated in methylcellulose medium in the presence of EPO alone and the resultant colonies were counted. (A) Total myeloid clonogenic progenitor cell (colony-forming unit, CFU) numbers per culture. (B) Total erythroid CFU numbers per culture.

Figure 6. The $CD34^+$ cells expanded by the EPOR-Mpl show the most balanced expression of multilineage surface markers. Human cord blood $CD34^+$ cells were transduced with the EPOwt, EPORGCR, or EPORMpl gene by the same retroviral vectors in Figure 3. After 14-day liquid culture with 10% FBS and 10 ng/ml EPO, the transduced cells were examined for the expression of glycophorin A (erythroid marker), CD15 (myeloid marker), and CD41 (megakaryocyte marker) by flow cytometry. The percentages of marker-positive cells are shown.

Figure 7. The gene-modified hematopoietic cells can be expanded by treatment

with EPO in vivo in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the retroviral vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice. The percentages of YFP-positive cells in the peripheral blood are shown in the EPOR-Mpl group (A) or the YFP control group (B). In each group, mice were divided into two subgroups; EPO-treated subgroup (n=6, 200 IU/kg, three times a week, closed bars) and EPO-untreated subgroup (n=4 or 6, open bars). The gray arrows in A and B indicate the week of EPO administration. The increase in YFP-positive cells in the EPO-treated mice was significant at week 10 (4 weeks after the initiation of EPO administration) (*, $p<0.05$).

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Table 1. Colony formation by human cord blood $CD34^+$ cells transduced with the EPO-driven SAGs.

	IL-3 (100 ng/ml) IL-6 (100 ng/ml) SCF (100 ng/ml)		EPO (20 ng/ml)	
Transgene	Number of colonies*	Provirus-positive colonies§	Number of colonies*	Provirus-positive colonies§
EPORwt-YFP	62±11	5/16 (31%)	15±3	15/16 (94%)
EPORGCR-YFP	54±8	6/16 (38%)	24±1	16/16 (100%)
EPORMpl-YFP	54±9	4/16 (25%)	31±6	15/16 (94%)
YFP	49±4	8/16 (50%)	12±1	9/16 (56%)
untransduced	53±4	ND	17±1	ND

* Colony number out of 200 cells is shown. Each value represents mean ± SD of triplicate culture.

§ Individual colony DNA was subjected to PCR for the proviral YFP and genomic β-actin sequences and the ratio of the provirus-positive colony number to the β-actin-positive colony number is shown.

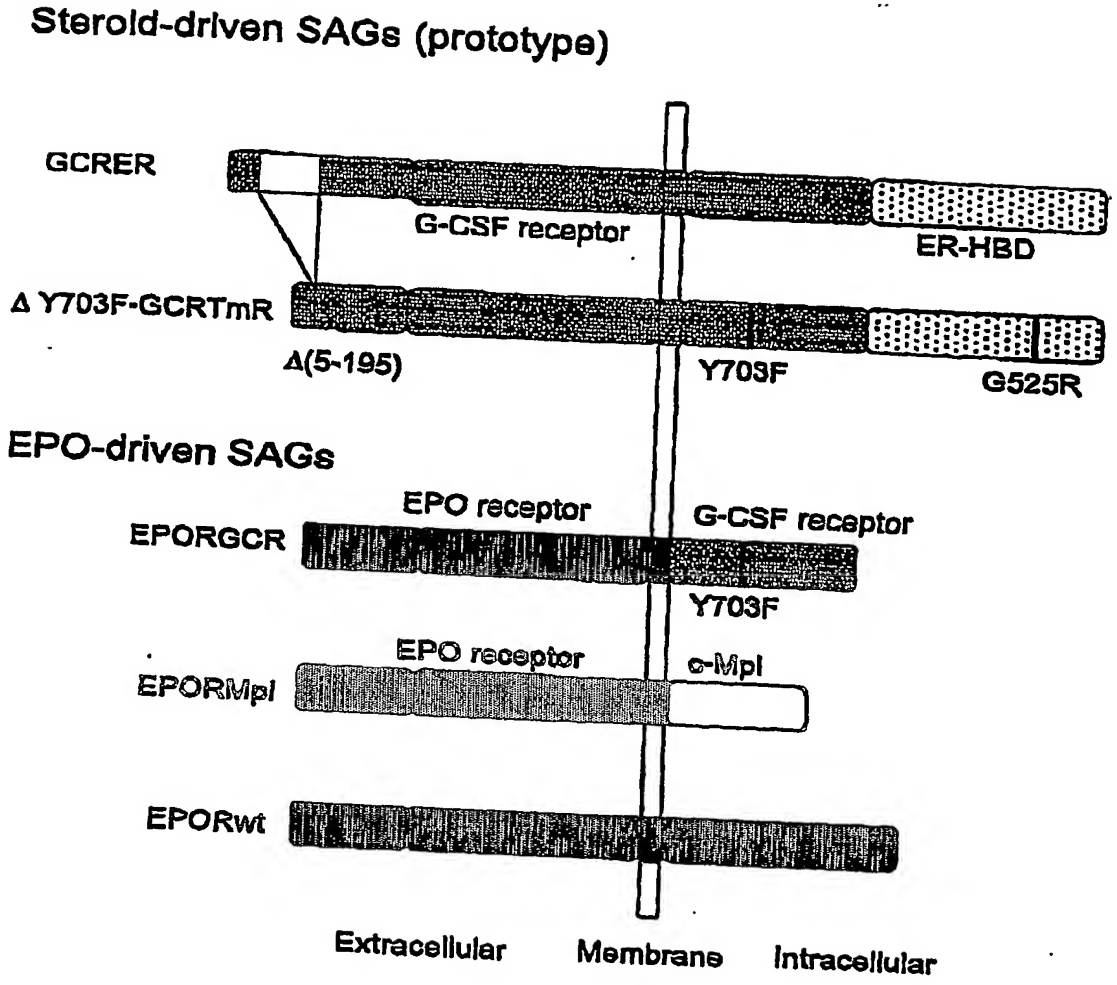
Sterold-driven SAGs (prototype)
GCRER
G-CSF receptor
ER-HBD
Δ Y703F-GCRTmR
Δ(5-195)
Y703F
G525R
EPO-driven SAGs
EPO receptor
G-CSF receptor
EPORGCR
Y703F
EPO receptor
c-Mpl
EPORMpl
EPORwt
Extracellular
Membrane
Intracellular

Figure 1

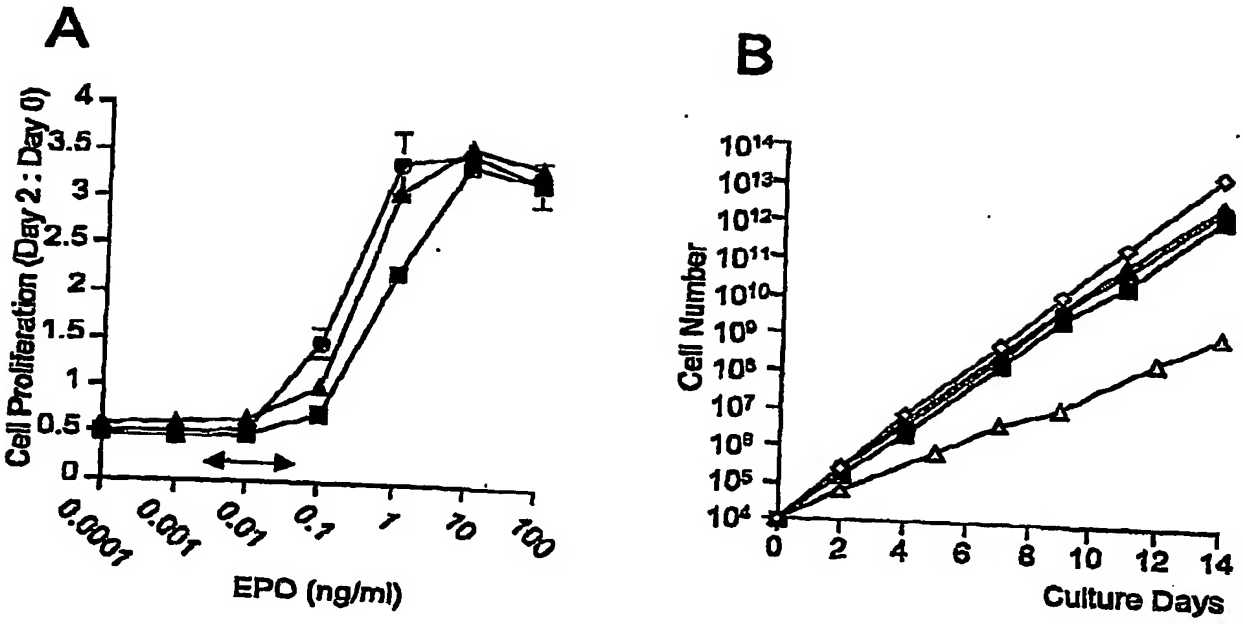
A
Cell Proliferation (Day 2 : Day 0)
4
3.5
3
2.5
2
1.5
1
0.5
0
0.0001
0.001
0.01
0.1
1
10
100
EPO (ng/ml)
B
Cell Number
10^{14}
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
0
2
4
6
8
10
12
14
Culture Days

Figure 2

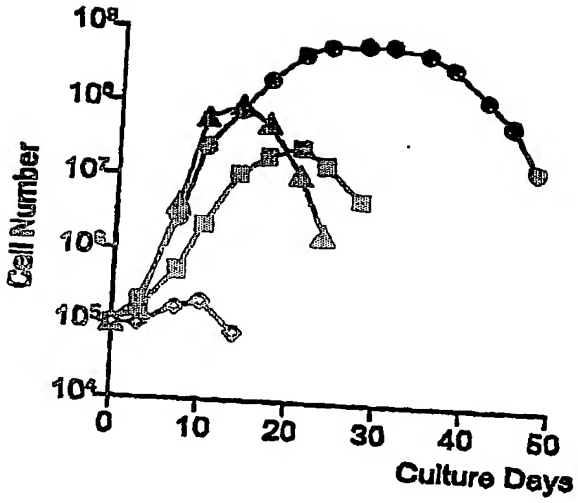

Figure 3

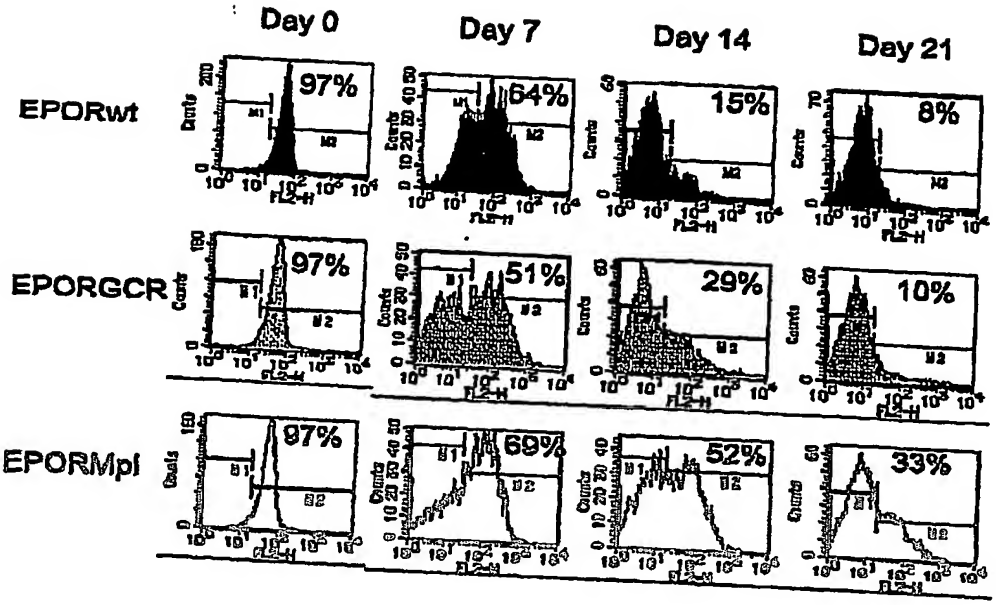
Day 0
Day 7
Day 14
Day 21
EPORwt
97%
64%
15%
8%
EPORGCR
97%
51%
29%
10%
EPORMpl
97%
69%
52%
33%

Figure 4

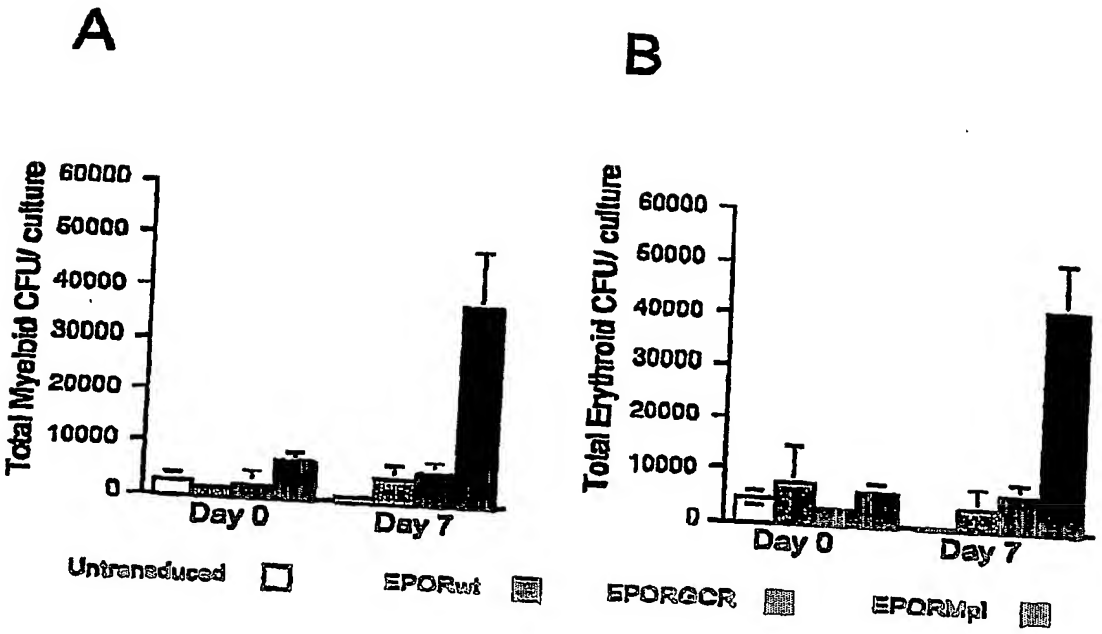
A
B
Total Myeloid CFU/ culture
60000
50000
40000
30000
20000
10000
0
Day 0
Day 7
Untransduced
EPORwt
Total Erythroid CFU/ culture
60000
50000
40000
30000
20000
10000
0
Day 0
Day 7
EPORGCR
EPORMpl

Figure 5

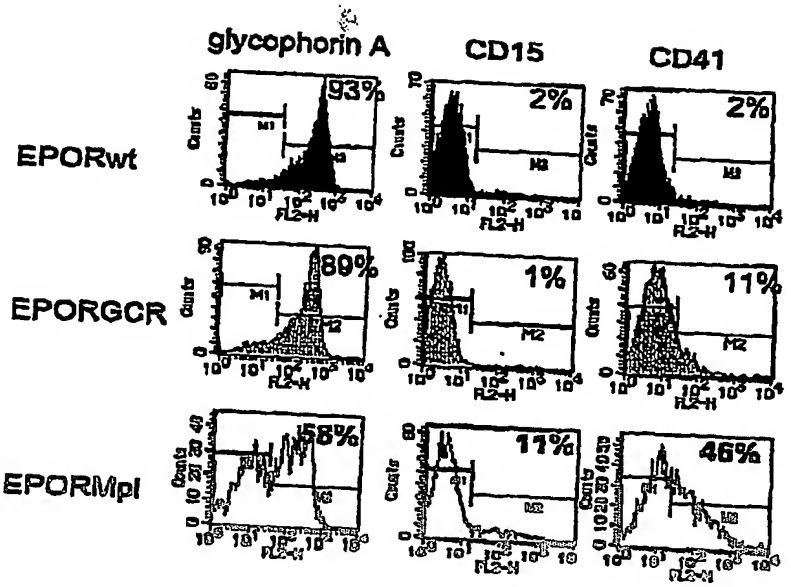
glycophorin A
CD15
CD41
EPORwt
EPORGCR
EPORMpl
93%
2%
2%
80%
1%
11%
58%
11%
46%

Figure 6

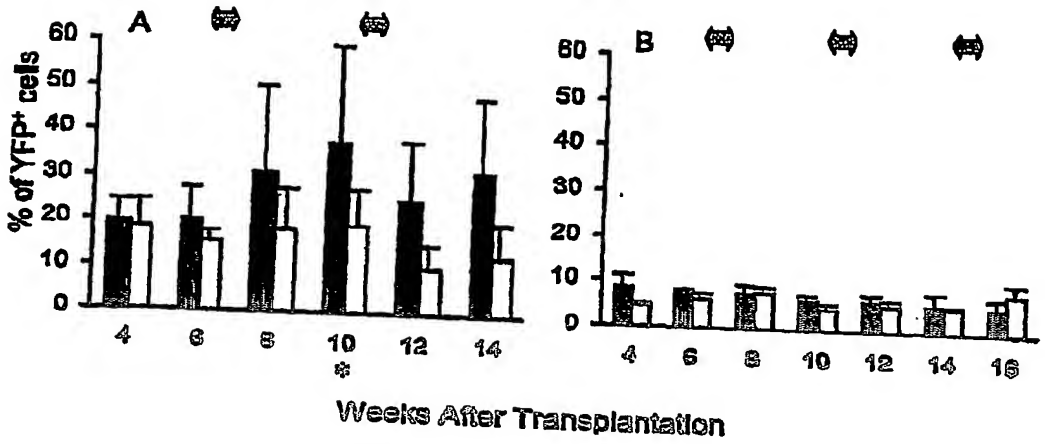
A
B
% of YFP+ cells
60
50
40
30
20
10
0
4
6
8
10
12
14
16
*
Weeks After Transplantation

Figure 7

Abstract

The successful engraftment of genetically modified hematopoietic stem cells without toxic conditioning regimen is a highly desirable goal for the hematopoietic stem cell (HSC) gene therapy. We have examined a novel HSC gene therapy method without marrow conditioning in a nonhuman primate model. Cynomolgus macaque $CD34^+$ cells were retrovirally marked and directly implanted into the limb bones (intra-bone marrow transplantation; iBMT) without marrow conditioning. After iBMT, 5-30% of colony-forming cells were gene-marked for one year. The marking level in the peripheral blood, however, remained low (<0.1%). The result indicates transplanted cells can engraft, but they do not expand as they do in myeloablated recipients. $CD34^+$ cells were then retrovirally engineered to express the selective amplifier gene (SAG), a chimeric gene consisting of the erythropoietin (EPO) receptor gene as a molecular switch and the thrombopoietin receptor (c-mpl) gene as a signal generator. The SAG-transduced cells were transplanted by iBMT. The SAG-transduced cells were increased by more than 20-fold (up to 7%) in the peripheral blood in response to EPO. The increase was polyclonal and observed in granulocytes, T- and B-lymphocytes. Thus, iBMT and SAG will allow clinically relevant in vivo gene transduction without marrow conditioning.

Introduction

The ability to positively select cells containing potentially therapeutic genes in vivo would represent an important tool for the clinical application of hematopoietic stem cell (HSC)-based gene transfer. This would circumvent low gene transfer efficiency into primate HSCs, which is the current limitation of this promising technology. The in vivo positive selection method would circumvent another major problem of HSC gene therapy: the necessity for myeloablation of recipients. It is associated with high systemic toxicity, or potential damage to marrow stroma possibly resulting in impaired engraftment (Plett 2002). Even without myeloablative conditioning, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels with this method.

Researchers have recently reported that bone marrow cells can engraft efficiently in mice without marrow conditioning by implanting cells directly into bone marrow cavity (intra-bone marrow transplantation, iBMT) in a murine model. (Zhong 2002) Using the iBMT method, human cord blood cells are also able to engraft efficiently in bone marrow of sublethally immunodeficient mice (Yahata 2003, Wang 2003). The iBMT method has, however, not been examined in primates.

We have previously developed the selective amplifier gene (SAG), a chimeric gene encoding the granulocyte colony-stimulating factor receptor as a growth-signal generator and the hormone-binding domain of the steroid receptor as a molecular switch (Ito 1997). Hematopoietic cells genetically engineered to express SAG can be expanded in a steroid-dependent manner in vitro and in vivo in mice and nonhuman primates (Kume 2003, Hanazono 2002). Here we have examined such expansion in the setting of nonhuman primate iBMT without marrow conditioning by using a new version of SAG, in which the erythropoietin receptor (as a molecular switch) and thrombopoietin receptor (as a signal transducer) are incorporated (Nagashima in press).

Results

Engraftment after iBMT

First, we have examined engraftment of gene-marked $CD34^+$ cells after iBMT. Cynomolgus $CD34^+$ cells were transduced with the non-expression retroviral vector PLI (containing a non-translated neo sequence). The transduction results are summarized in Table 1. Instead of the conventional transplantation method (intravenous injection), we injected transduced $CD34^+$ cells directly into bone marrow cavity of four proximal limb bones (the femur and humerus) after washing the cavity with PBS (Fig. 1). Any conditioning treatment such as irradiation was not conducted prior to transplantation. After transplantation, we took marrow cells and placed the cells in methylcellulose. The resulting clonogenic colonies (CFU) were examined for the provirus by PCR. Five to 30% of colonies were positive for the provirus and this high marking level persisted over one year. Thus, long-lived progenitor cells did engraft by iBMT. Interestingly, the provirus in CFU in non-implanted bone marrow was detectable within two weeks after transplantation, and the marking levels were comparable to those in implanted bone marrow. Thus, transplanted cells relocated from an implanted bone to another at early time points. The peripheral blood cells were also examined for the provirus by quantitative PCR (Fig. 2). The marking levels were, however, found to be very low (< 0.1%). Taken together, transplanted cells can engraft but they do not proliferate as they do in myeloablated recipients.

To proliferate engrafted cells, we administrated G-CSF and stem cell factor (SCF) to the animals for five days. The transient increase in the fraction of the vector-containing cells was observed in the peripheral blood (Fig. 2). The increase may be explained by mobilization of transduced cells from the bone marrow into the periphery.

Expansion by SAG

We constructed a retroviral vector expressing the SAG; a chimeric gene of the human erythropoietin (EPO) receptor gene (as a molecular switch) and human c-Mpl gene (as a signal trenaducer) (Nagashima in press). Cells genetically engineered to express the SAG will proliferate in an EPO-dependent manner. We transduced cynomolgus $CD34^+$ cells with the SAG vector and implanted the cells into non-conditioned autologous recipients by iBMT. The transduction results are summarized in Table 1.

In one animal (Fig. 3a), subcutaneous EPO administration triggered a striking elevation in marking levels (up to 7.4 %) in the peripheral blood. The in situ PCR for the proviral sequence showed many transduced cells in the peripheral blood mononuclear cells at this time point (Fig. 4a). After cessation of EPO administration, the marking level rapidly fell down to <0.1%. We resumed subcutaneous EPO administration at the same dose twice daily. The second trial of EPO treatment resulted in the elevation in the marking levels (up to 4.6%). EPO administration caused mild increase in hematocrit but it was successfully treated by occasional phlebotomy. No other adverse effects were observed.

In another animal (Fig. 3b), the SAG-transduced cells increased following transplantation even without EPO administration. The increase might be attributable to endogenous EPO elevation, since the animal was anemic during the period. When the anemia was ameliorated and the marking level fell down, we started intravenous EPO administration at a dose of 200 IU/kg three times weekly for two weeks. The marked cells in the peripheral blood did not increase despite the EPO administration. The intravenous administration 3 times a week seemed insufficient to proliferate the transduced cells. We then started subcutaneous administration of EPO at the same does once a day for 60 days and then twice a day for further 30 days. The SAG-positive cells then expanded by more than 20 folds. The cells declined to the basal level after

discontinuation of EPO. We again tried subcutaneous EPO administration, but the SAG-positive cells were no longer increased but cleared from the periphery within a month after the administration, most likely due to CTL induction to the human EPO receptor moiety included in the SAG (see below).

Dual marking

We have then compared the effect of the SAG vector to non-SAG vector within, rather than between, individual animals. Cytokine-mobilized peripheral blood $CD34^+$ cells were harvested and split into two equal aliquots. One aliquot was transduced with the SAG vector and the other with the control non-expression vector (PLI). Both aliquots were mixed and transplanted by iBMT without marrow conditioning. The animal received EPO subcutaneously once a day from the next day of transplantation and in vivo marking levels derived from two populations were examined by quantitative PCR.

Cells containing the SAG vector were increased by two logs in the peripheral blood in response to EPO, although cells containing the non-expressing vectors remained at low levels (Fig. 3c). However, SAG-containing cells were rapidly cleared within 1 month posttransplantation to undetectable levels. The SAG-vector marking level became even lower than the non-expression vector marking level. Such quick drop in SAG-transduction levels in the periphery was also observed in another monkey (BMR9, data not shown).

We have examined the serum concentration of human EPO in all animals receiving human EPO, but the concentration did not drop, since we gave cyclosporin A to all the animals receiving EPO. Thus, humoral response to the human EPO was not a reason of the decrease in the SAG-transduced cells. On the other hand, the current SAG is a chimeric gene f human origins (the human EPO receptor and human c-Mpl). We have detected CTLs to the SAG and to the human EPO receptor in BMR9 that received

SAG (data not shown). The CTL activity to the human EPO receptor moiety was almost comparable to the entire SAG. Thus, CTL induction to the human EPO receptor moiety is most likely a reason for the clearance of SAG transduced cells in some animals. This may also explain why the SAG-transduced cells were cleared after the second trial of EPO administration in BMR6 (Fig. 3b).

Multilineage and polyclonal marking

We sorted granulocytes, T- and B-lymphocytes from BMR8 (Fig. 3a) by flow cytometry and DNA of sorted cell was subjected to quantitative PCR for the provirus. The provirus-containing fraction in granulocytes was 6%, and that in B- and T-lymphocytes was both 2% (Fig. 4b). Thus, transduced cells expanded in multilineage including T- and B-lymphocytes. An analysis of integration sites using the linear amplification mediated (LAM)-PCR method (Schmidt 2003) indicates that multiple clones contributed to the increase in transduced cells in response to EPO (Fig. 4c).

Discussion

Our results indicate that transplanted $CD34^+$ cells can engraft by iBMT without a conditioning regimen, but that they do not expand as they do in myeloablated recipients. When $CD34^+$ cells were genetically engineered to express SAG, the cells could proliferate (by more than 20-fold) by treatment with EPO after iBMT even without marrow conditioning. The marking levels reached up to 7% in the peripheral blood. The cynomolgus marking levels after myeloablative intravenous transplantation in our institution are 10% at best. The present marking results after iBMT without marrow conditioning is thus comparable to the marking levels after the conventional myeloablative transplantation. In the current study, four proximal limb bones (the femur and humerus) were used for transplantation. If other bones such as the iliac bone (which contains more marrow) are used, marking levels may be further improved. Expansion of SAG-transduced cells was seen in three lineages of leukocytes; granulocytes, B-, and T-lymphocytes. The Mpl-signal generated by SAG may work even in lymphocytes. In fact, B-lymphocytes were shown to be increased by the activated c-Mpl in a canine transplantation model (Neff 2002). Multiple clones were found to contribute to gene-marked cells, demonstrated by highly sensitive LAM-PCR method, clearly indicating no outgrowth of a single or oligo-clonal population.

The best marking result after myeloablative transplantation in nonhuman primates has been 60% of gene-modified cells in the periphery (Kelly 2003). Other groups documented the marking levels of 25-29% in the periphery in nonhuman primates (Takatoku 2001, Horn 2002, Hemani 2003). The marking results with nonablative conditions of reduced intensity showed generally lower marking levels in the periphery. Rozenzweig et al. reported 10-15% at 4 months posttransplantation with 320-400 cGy total body irradiation (Rosenzweig 1999). Huhn et al. reported 12% at best with 500 cGy total body irradiation (Huhn 1999). On the other hand, without marrow conditioning, Malech et al. and Dunbar et al. reported that much less than 0.1% of cells

were marked (or corrected) after $CD34^+$ cell gene therapy of chronic granulomatous disease and Gaucher disease (Malech 1997, Dunbar 1998). From these lines of observation, researchers have believed that myeloablation (or at least conditioning of reduced intensity) was necessary for engraftment of transplanted cells.

However, it has recently been shown that, in a murine transplantation model, hematopoietic stem cells can engraft in bone marrow even without marrow conditioning by iBMT (Zhong 2002). Cells may be more efficiently engrafted in bone marrow by iBMT rather than the intravenous transplantation (Yahata 2003, Wang 2003). Our data suggest that gene-marked hematopoietic cells can indeed engraft in bone marrow of nonablated monkeys after iBMT without marrow conditioning, but that transplanted cells does not proliferate in these animals presumably due to the steady state of hematopoiesis, additional hematopoiesis not being required. This is the case in a murine iBMT model (Zhong 2002). Interestingly, although transplanted cells stay quiescent in bone marrow of nonablated monkeys, they were detected in non-implanted bones within two weeks after transplantation. Thus, translocation of transplanted cells from an implanted bone to another occurs at early time points after transplantation. Similar early translocation posttransplantation has also been reported in a human-mouse xenotransplantation model (Kushida 2002, Yahata 2003, Wang 2003).

Unfortunately, CTLs were generated against SAG, especially against the human EPO receptor moiety, in some animals, resulting in the failure to respond to EPO. Although the difference in the amino acid sequence of EPO is quite limited between humans and macaques, the human EPO receptor could be still immunogenic to macaques. In non-myeloablated animals, immune responses against transgene products recognized as foreign can indeed be a major obstacle to long-term persistence of gene-modified cells in vivo (Riddell 1996). In human clinical situations, however, CTLs should not take place against SAG, since it is made of allogeneic genes.

Our results suggest that expansion of transduced cells is transient, as is the case with

chimeric genes containing c-*mpl* as a signal transducer by other groups (Neff 2002). The method may not result in selection of transduced cells at the level of HSCs, but within the differentiated progeny of transduced HSCs. In order to obtain clinically relevant effects, repeated EPO administration might be required. EPO is a safe drug and can be administered repeatedly with minimal adverse effects. Polycythemia is the only side effect observed in the present study. It can, however, be treated by phlebotomy easily and safely. Therapeutic effects can also result from continuously elevated levels of endogenous EPO in patients with thalassemia for instance. When anemia is ameliorated and endogenous EPO levels return to physiological levels, then the positive selection system is "automatically" turned off. This system may be convenient. In conclusion, HSC gene therapy without marrow conditioning is feasible by iBMT with SAG included in a vector together with a therapeutic gene such as the thalassemia gene.

Methods

Animals

Young cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of Tsukuba Primate Center and the guide principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The protocol of experimental procedures was approved by the animal welfare and animal care committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Collection of cynomolgus $CD34^+$ cells

The cynomolgus monkeys received recombinant human (rh) SCF (50 μg/kg; Amgen, Thousand Oaks, CA) and rhG-CSF (50 μg/kg; Chugai, Tokyo, Japan) as daily subcutaneous injections for 5 days. Peripheral blood (PB) cells or bone marrow (BM) cells were then collected by leukapheresis or by aspiration from iliac bones (Hanazono 2002). From the harvested PB and BM cells, the leukocyte cell fraction was obtained by red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan).

Enrichment of $CD34^+$ cells was performed using magnet beads conjugated with anti-human CD34 monoclonal antibody (clone 561) (Dynal, Lake Success, NY, USA). The purity of $CD34^+$ cells ranged from 90 to 95% as assessed with another monoclonal anti-CD34 antibody (clone 563; PharMingen, San Diego, CA). Mean CFU enrichment was 48-fold as assessed by colony-forming progenitor assays performed before and after enrichment.

Retroviral transduction

We used SAG retroviral vector containing a chimeric gene of EPOR and c-Mpl

(Nagashima in press) and PLI retroviral vector which is a non-expression vector containing the *neo* sequence which could be detected by PCR (Heim 2001). The titers of the viral supernatants used in these studies were both $1x10^6$ particles per ml, as assessed by RNA dot blot.

$CD34^+$-enriched cells were cultured at a starting concentration of 1-5 x 10^5 cells/ml in fresh vector supernatant of PLI or SAG in the presence of SCF, thrombopoietin (TPO) (Kirin, Tokyo, Japan) and Flt-3 ligand (FL) (RDI, Flanders NJ) at the 100 ng/ml each, in dishes coated with RetroNectin (Takara, Shiga, Japan) according to the manufacturer's instruction (SCF/TPO/FL/FN). Every 24 hours, nonadherent cells were collected, spun down, resuspended in fresh vector supernatant and cytokines, and then returned to the same RetroNectin-coated dishes. After 96-hour transduction, cells were washed and continued in culture for two additional days in the same RetroNectin-coated dishes in the presence of SCF alone (Takatoku 2001).

Intra-bone marrow transplantation

Cynomolgus monkeys were anesthetized. Two needles were inserted into both ends of the humerus and femurs. A syringe containing 50 ml of phosphate-buffed saline (PBS) was connected to the needle and other needle was connected to an empty syringe. The PBS was flushed gently from one syringe to another through the marrow cavity twice (Fig. 1). Then gene-modified cells (suspended in 1 ml of the PBS containing 10% autologus serum) were injected into the marrow cavity and the needle holes were sealed with bone wax. We measured the internal pressure in the marrow cavity during the injection procedure and carefully performed iBMT without inflicting extra-pressure to the marrow cavity (data not shown). All animals did not suffer from neutropenia, thrombocytopenia, infection, or pulmonary embolism without any morbidity.

After transplantation, EPO (Chugai, Tokyo, Japan) was administered to animals receiving SAG at a dose of 200 IU/kg once or twice daily subcutaneously as otherwise

indicated. Cyclosporine A was administrated to the animals a week prior to the EPO administration to prevent the development of anti-human EPO antibody.(Schuumann 2001)

Clonogenic hematopoietic progenitor assay

Cells were plated in 35 mm petri-dish in 1 ml of alpha-MEM containing 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan) supplemented with 100 ng/ml recombinant human interleukin-3 (PeproTech, Rocky Hill, NJ), 100 ng/ml recombinant human interleukin-11 (PeproTech), 100 ng/ml recombinant human SCF (Biosource, Camarillo CA), 2 U/ml rhEPO (Roche, Basel, Switzerland), 20% FCS, 1% deionized BSA, $5x10^{-5}$M 2-mercaptoetanol (Sigma), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). For colony formation from SAG-transduced cells, rhEPO was removed from the media. After incubation for 14 days at 37°C with 5% CO_2, colonies containing greater than 50 cells were counted using an inverted light microscope. Experiments were conducted in triplicate.

Quantitative PCR

Genomic DNA was extracted using the QIAamp Blood Kit (Qiagen, Chatsworth, CA, USA). PCR amplification and analysis of the PLI and SAG genes were performed by using a quantitative real-time PCR assay. DNA (250 ng) was amplified in triplicate with neo-specific primers (5'-TCC ATC ATG GAT GCA ATG CGG C-3' and 5'-GAT AGA AGG CGA TGC GCT GCG AAT CG-3') for PLI and the specific primers for SAG (5'-GAC GCT CTC CCT CAT CCT CGT-3' and 5'-GAG GAC TTG GGG AGG ATT TCA-3'). Standards consisted of DNA extracted from producer cell lines (which have two and four copies of the provirus) serially diluted with control cynomolgus genomic DNA. Negative controls consisted of DNA extracted from peripheral blood nucleated cells. A beta-actin-specific primers (5'-CCT ATC AGA

AAG TGG TGG CTG G-3', 5'-TTG GAC AGC AAG AAA GTG AGC TT-3) was used to certify equal loading of DNA per reaction.

Reactions were run using the Qiagen SYBR Green PCR Master Mix (Qiagen) on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the following conditions: 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 62°C for 30 sec, 72°C for 30 sec and 83°C for 0.15 sec. All quantitative PCR was certified each time to yield linear amplifications in the range of the intensity of the positive control series (0.01-100%, correlation coefficient > 0.98). For calculating the transduction efficiencies, the Ct value of the vector sequence was normalized based on the Ct value of the internal control beta-actin sequence on the same sample as directed in the manufacturer's protocol. Calculated gene marking percentages are adjusted for the assumption that the peripheral blood cells contain only one copy of the corresponding vector per cell.

Colony PCR

At day 14, colonies containing greater than 50 cells were counted, and individual colonies were plucked into 50 µl of distilled water, digested with 20 µg/ml proteinase K (Takara) at 55°C for 1 hour followed by 99°C for 10 min, and assessed for vector SAG or non-expressing vector PLI sequence by nested PCR. The outer primer sets were the same as used in the quantitative PCR described above. Amplification conditions for outer PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 20 cycles. The outer PCR products were purified by the MicroSpin S-400 HR Columns (Amersham, Piscataway, NJ, USA). The inner primer set was 5'-CCA CCC CTA GCC CTA AAT CTT ATG-3' and 5'-GGT GGT TCA GCA TCC AAT AAG G-3' for SAG vectors, and 5'-ATA CGC TTG ATC CGG CTA CCT G-3' and 5'-GAT ACC GTA AAG CAC GAG GAA G-3' for PLI vectors. Amplification conditions for inner PCR were 95°C for 1 min, 54 for 1 min, and 72°C for 2 min with 20 cycles. Simultaneous

PCR for beta-actin sequence was also performed to document DNA amplification of the sample on each colony. The primer set for beta-actin was the same as used in the quantitative PCR described above. Amplification conditions for beta-actin PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 30 cycles. The sizes of the final PCR products were 206, 483, and 232 bp for SAG, non-expressing PLI vector, and beta-actin sequences, respectively. PCR products were separated on 2% agarose gels. The transduction efficiency of CFU was calculated by dividing the number of positive colonies for vector sequence by the number of positive colonies for beta-actin sequence.

In situ PCR

In situ detection of transplanted cell progeny was performed by amplifying the SAG sequence as previously reported.(Hasse 1990) Peripheral blood cells were spun down to slide glasses. The primer sequences were the same as used for the quantitative PCR described above. The reaction mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μM of each SAG primer, 4.5 mM $MgCl_2$, PCR buffer (Mg^{2+} free), and 4 U Takara Taq DNA polymerase (Takara). Slides were covered with the Takara Slide Seal for in situ PCR (Takara). PCR was performed using the PTC100 Peltier Thermal Cycler (MJ Research, Watertown, MA) with the following conditions; 94°C for 1 min and 55°C for 1 min with 15 cycles. After amplification, cover seals were lifted off and slides were washed with PBS at room temperature 3 times each for 5 min.

The digoxigenin incorporated-DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (Dako, Glostrup, Denmark) followed by histochemical staining. Briefly, slides were incubated at 37°C for 3 h with the HRP-conjugated rabbit F(ab') anti-digoxigenin antibody solution diluted (1:100) in blocking solution (2% bovine serum albumin and 5% normal horse serum in PBS), and rinsed with PBS at room temperature 3 times each for 5 min. Slides were

then stained for HRP using the Vector SG substrate kit (Vector, Burlingame, CA). The reaction was terminated by transferring slides into a water bath upon development of a dark blue or black color (usually requiring 3-10 min to develop the signal). Finally, sections were counterstained with the Kernechtrot that stains nucleotides, washed with water, mounted in glycerol, and examined under a light microscope.

Southern blot

Ten microgram of genomic DNA was digested with *KpnI* (Takara), which cuts once within each viral long terminal repeat. The DNA fragments were transferred to Hybond-N+ (Amersham, Cleveland, OH) and hybridized with a radiolabeled viral packaging signal-specific probe generated by PCR using following primers: forward primer 5'-TCT GTA CTA GTT AGC TAA CTA GCT CTG TA-3' and reverse primer 5'-AAG ACC TTG ATC TTA ACC TGG GTG ATG A-3'. Radiolabelling of the probes was done using an oligolabelling kit (Pharmacia, Piscataway, NJ).

Flow cytometric sorting

We used the FSC/SSC profile (forward and side scatter) to sort granulocyte subsets. Anti-CD3 and anti-CD20 was used to sort the T- and B-lymphocyte subset, respectively. The cells were sorted using a FACS Vantage (Becton Dickinson) or EPICS ELITE (Beckman Coulter) cell sorter, each equipped with an argon-ion laser. Data acquisition and analysis were performed using the CellQuest or EXPO2 software (Beckman Coulter), respectively.

Linear amplification mediated (LAM)-PCR

LAM-PCR was performed as previously described (Schmidt 2003). The genomic-proviral junction sequence was preamplified by repeated primer extension using 0.25 pmol of vector-specific, 5'-biotinylated primer LTR1 (5'-AGC TGT TCC

ATC TGT TCT TGG CCC T-3') with Taq polymerase (2.5U; Qiagen) from 100 ng of each sample DNA. As described above, 100 cycles of amplification were performed with addition of fresh Taq polymerase (2.5 U) after 50 cycles. Selection of biotinylated extension products was performed with 200 µg of magnetic beads according to the manufacturer's instructions (Dynal). The samples were incubated with Klenow polymerase (2 U; Roche), dNTPs (300 µM; Pharmacia, Uppsala, Sweden), and random hexanucleotide mixture (Roche) in a volume of 20 µL for 1 hour at 37°C. Samples were washed on the magnetic particle concentrator (Dynal) and incubated with TasI endonuclease (4 U in 20 µL; Hybaid-AGS, Middlesex, UK) for 1 h at 55°C. After an additional wash step, 100 pmol of a double-stranded asymmetric linker cassette and T4 DNA ligase (6 U; New England Biolabs, Beverly, MA, USA) were incubated with the beads in a volume of 10 µL at 16°C overnight. Denaturing was performed with 5 µL of 0.1 N NaOH for 10 min at room temperature. Each ligation product was amplified with Taq polymerase (5 U; Qiagen), 25 pmol of vector-specific primer LTR2 (5'-GAC CTT GAT CTG AAC TTC TC-3'), and linker cassette primer LC1 (5'-GAC CCG GGA GAT CTG AAT TC-3'), using the amplification conditions described above. Of each PCR product, 0.2% served as a template for a second, nested PCR with internal primers LTR3 (5'-TCC ATG CCT TGC AAA ATG GC-3') and LC2 (5'-GAT CTG AAT TCA GTG GCA CAG-3') at identical conditions. Of this final product, 80% were separated on a 2% agarose gel.

CTL assay

PB mononuclear (PBMC) cells were isolated from the animal (BMR9) receiving SAG and PLI vectotrs. Stimulator cells were prepared by transducing $4x10^6$ autologous bone marrow stromal cells with SAG vector. Stimulator cells were washed twice with RPMI1640 containing 10% FCS followed by irradiatation at 3,000 cGy using an X-ray source. Freshly isolated autologous PBMCs were mixed with the

stimulator cells at the ratio of 4:1 and cultured in RPMI1640 containing 10% FCS and 20 IU/ml human recombinant IL-2 for 13 days and served as effector cells.

The autologous bone marrow stroma cells were transduced with the SAG, human EPO receptor full length cDNA, and PLI and were used as target cells. To label target cells with ^{51}Cr, target cells ($1x10^6$) were resuspended in 100 µl of RPMI1640 containing 10% FCS, and 100 µl of ^{51}Cr (PerkinElmer Life Sciences, Boston, MA) was added to each cell suspension (final 0.1 mCi of $^{51}Cr/10^6$ cells). Cells were then incubated at 37°C for 1 hour with gentle shaking. Cells were subsequently washed twice and resuspended in 1 ml of the medium. The number of viable cells was counted and adjusted at the cell density of $5x10^5$/ml. The viability of ^{51}Cr-labeled cells was greater than 80% as determined by trypan blue staining.

CTL activity was determined by lysis of target cells as described below. A hundred µl of effector cells suspended in RPMI1640 containing 10% FCS were mixed with $5x10^3$ autologous target cells suspended in 100 µl RPMI1640 containing 10% FCS in U-bottom 96-well culture plates to yield effector to target cell (E/T) ratios of 100:1, 50:1, 25:1, 12.5:1, 6.4:1 and 3.2:1. The plates were placed in a 37°C, 5%CO_2 incubator for 4 h. Following incubation, plates were centrifuged at 700 rpm for 5 min and the supernatants were collected from each well. ^{51}Cr release was measured using a gamma counter (Aloka, Tokyo, Japan). Spontaneous ^{51}Cr release was determined as the count derived from supernatant from a well containing the target cells alone. The maximum release was determined as that corresponding to target cells treated with NP-40 (Sigma) before the final centrifugation. The CTL activity was measured in triplicate and was expressed as percentage lysis using the following formula.

$$\% \text{ Lysis} = \frac{(\text{cpm of effector + target}) - (\text{cpm of spontaneous release})}{(\text{cpm of maximum release}) - (\text{cpm of spontaneous release})} \times 100$$

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Table 1. Ex vivo transduction

Monkey no.		Target cell source	Vector	No. of infused CD34+ cells/kg	% PCR positiv CFUs (provirus/beta -actin)
2048	BMR4	BM	PLI	13.5×10^7	34/46(73.9%)
3053	BMR5	PB	PLI	2.10×10^7	49/78(62.8%)
3047	BMR6	PB	SAG	8.38×10^7	20/35(57.1%)
9042	BMR8	PB	SAG	3.08×10^7	11/21(52.4%)
8058	BMR9	PB	SAG	4.33×10^6	11/43(25.6%)
			PLI	3.20×10^6	9/42(21.4%)

PLI, non-expression vector

Figure 1. The iBMT method

a. Gentle washing of bone marrow cavity with PBS (photo).

b. dino (schemaric diagram)

Figure 2. Successful engraftment of transplanted cells but low levels of proliferation after iBMT without marrow conditioning.

a. BMR4

b. BMR5

Figure 3. Successful expansion of SAG-transduced cells in response to EPO after iBMT.

a. BMR8

b. BMR6

c. Dual genetic marking study

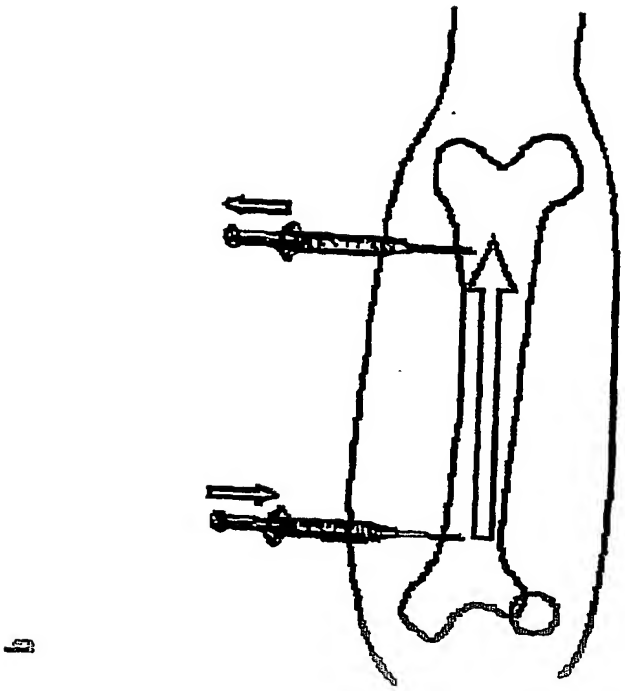
Figure 4. High-level, multiliniage, polyclonal marking with SAG in the peripheral

blood after iBMT without marrow conditioning.

a. In situ PCR for the provirus.

b. Lineage analysis by semi-quantitative PCR.

c. Clonal analysis by LAM-PCR



b

a

Fig 1

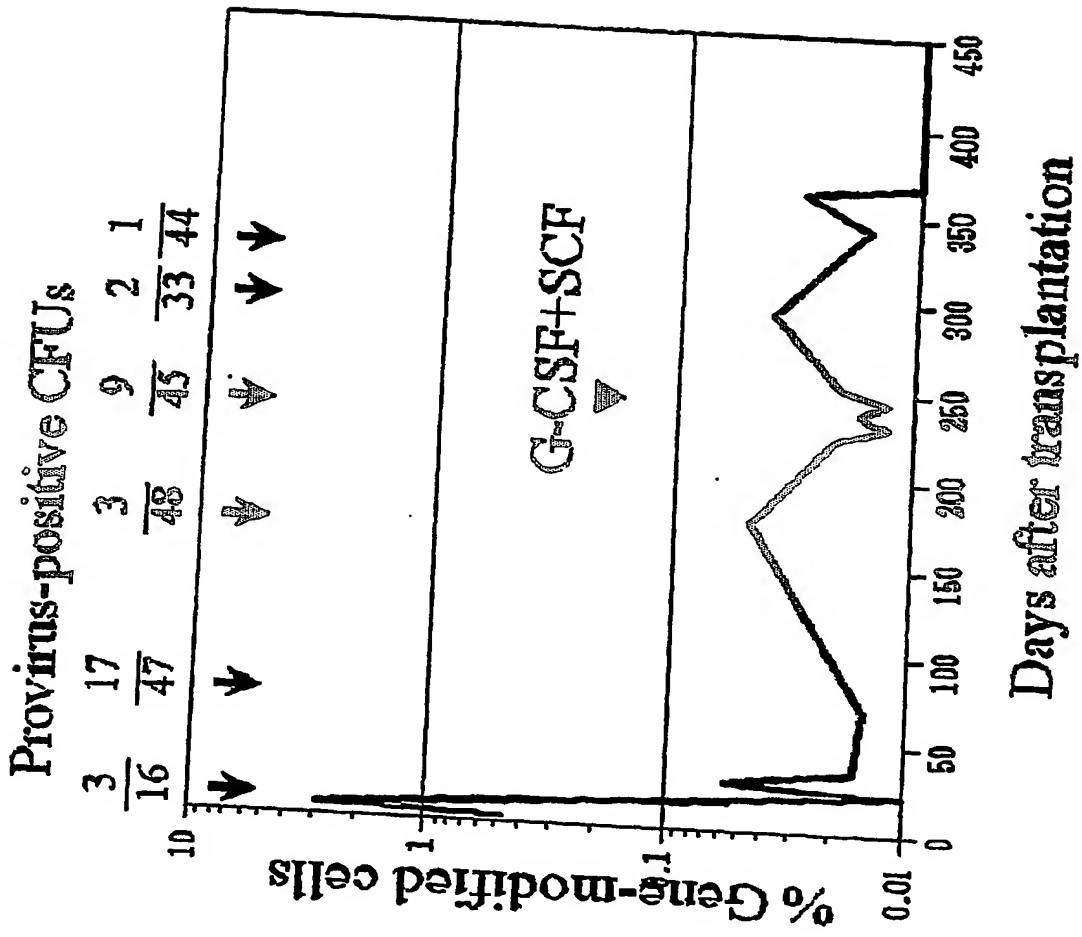

Provirus-positive CFUs
3/16 17/47 3/48 9/45 2/33 1/44
G-CSF+SCF
% Gene-modified cells
10
1
0.1
0.01
0
50
100
150
200
250
300
350
400
450
Days after transplantation

Fig 2a

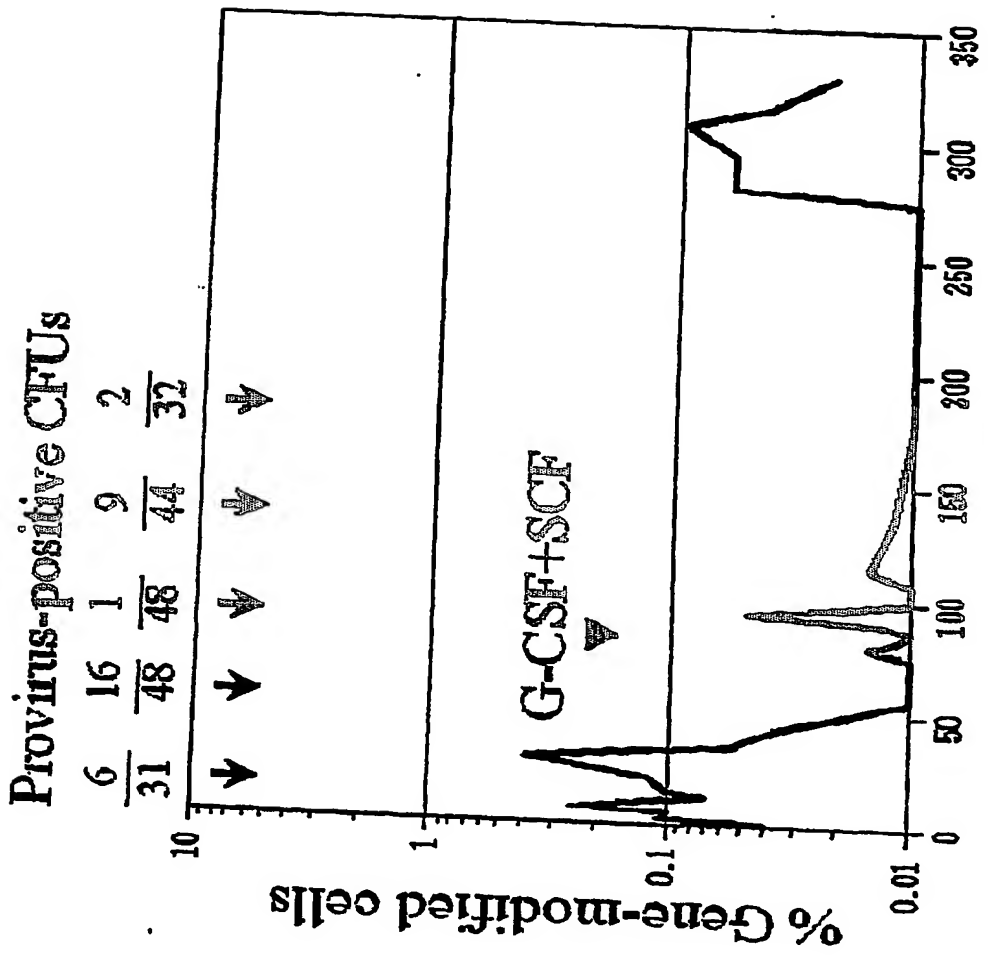

Provirus-positive CFUs
6/31
16/48
1/48
9/44
2/32
G-CSF+SCF
% Gene-modified cells
10
1
0.1
0.01
0
50
100
150
200
250
300
350
Days after transplantation

Fig 2b

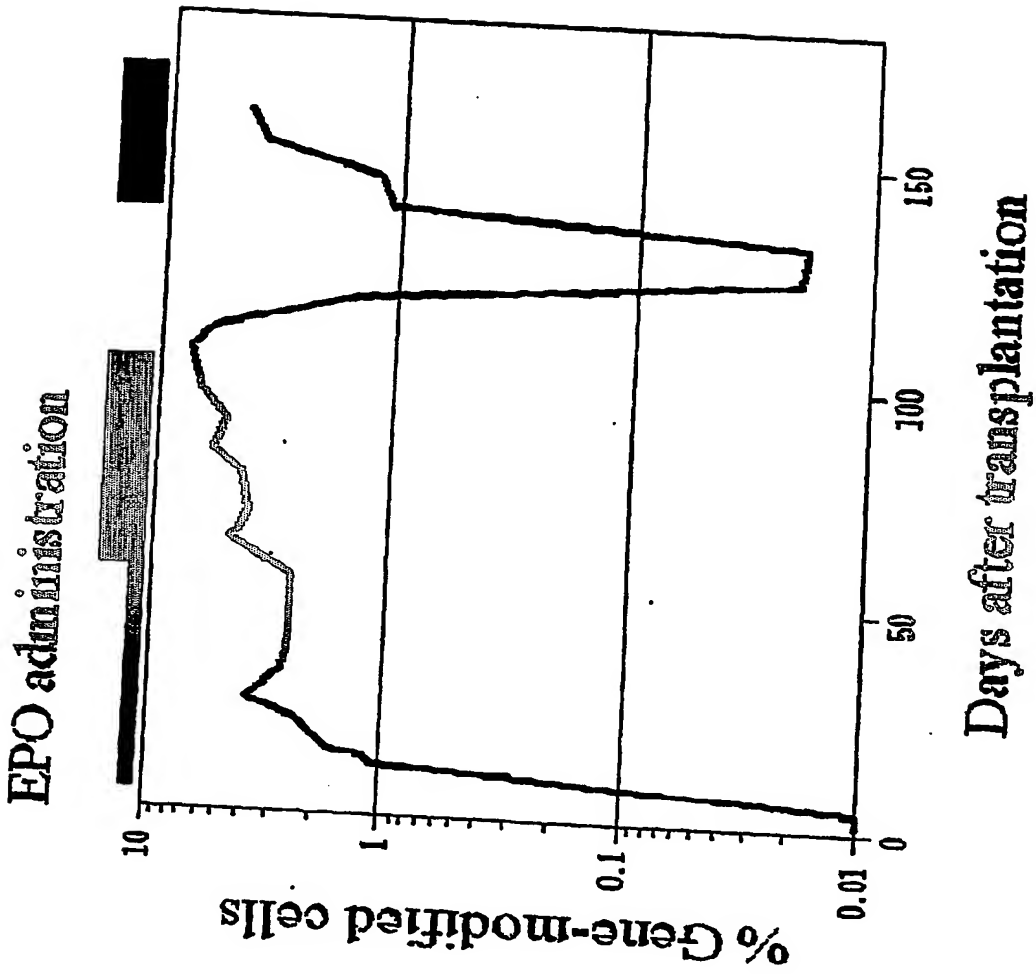

EPO administration
% Gene-modified cells
10
1
0.1
0.01
0
50
100
150
Days after transplantation

Fig 3a

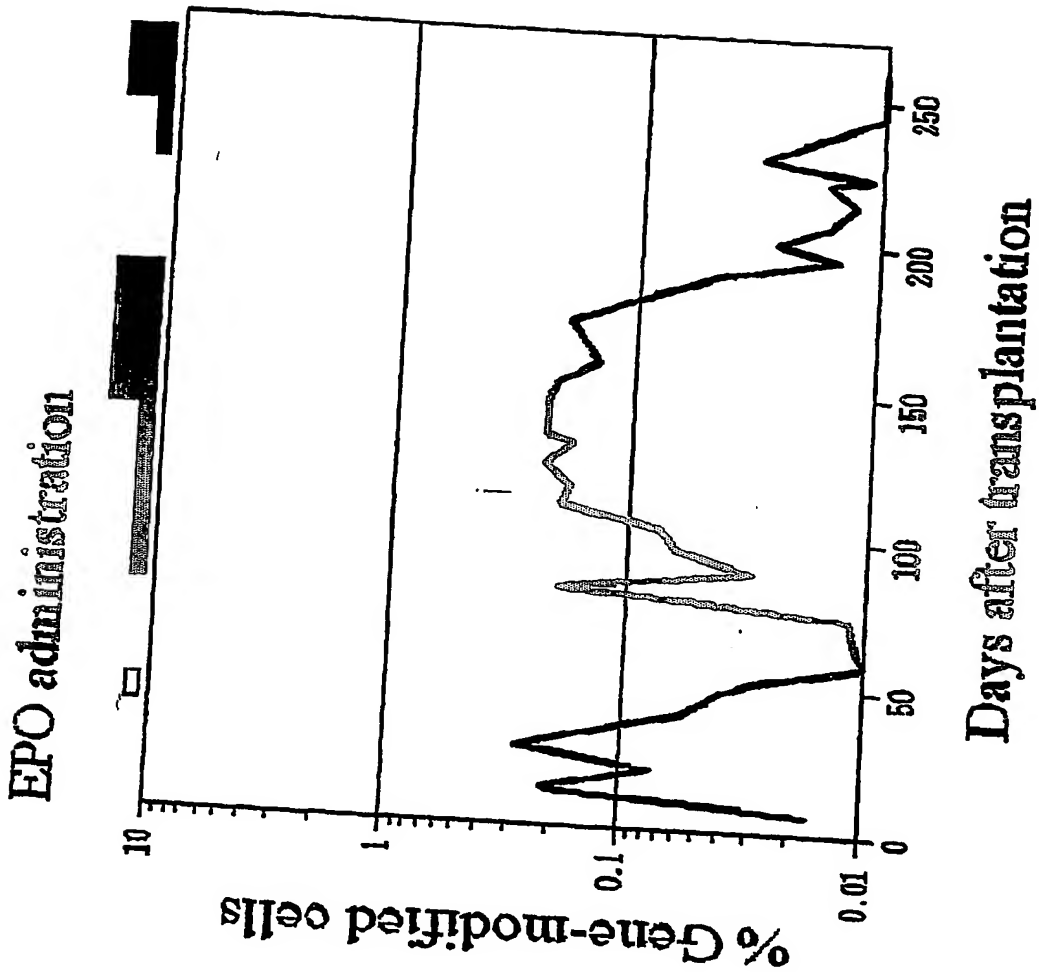

EPO administration
% Gene-modified cells
10
1
0.1
0.01
0
50
100
150
200
250
Days after transplantation

Fig 3b

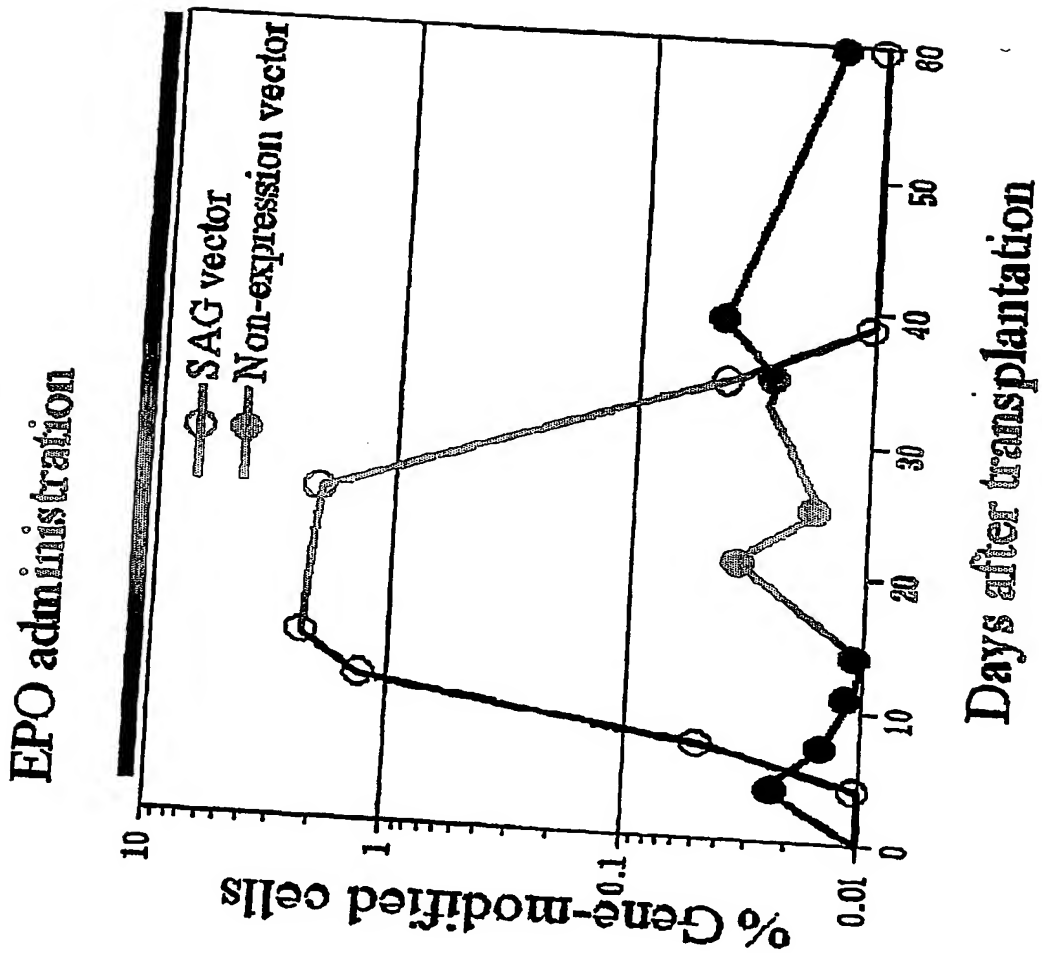

EPO administration
SAG vector
Non-expression vector
% Gene-modified cells
10
1
0.1
0.01
0
10
20
30
40
50
60
Days after transplantation

Fig 3c

Fig 4a

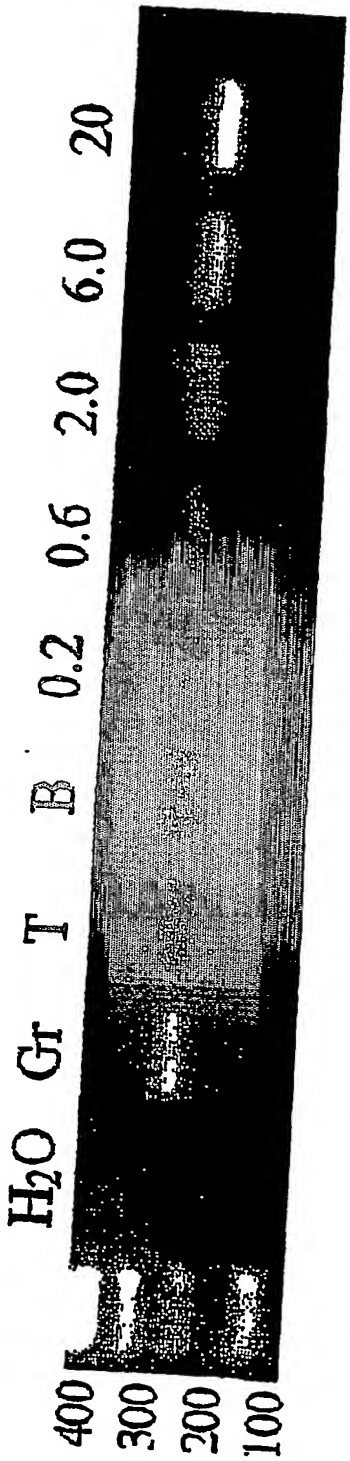


Fig 4b

Other embodiments

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

Claims

1. A method for transplanting lymphohematopoietic cells into a mammal, wherein the method comprises the step of injecting cells into a bone marrow cavity, and wherein the cells have an exogenous gene encoding a receptor that induces cell proliferation in response to ligand binding.

2. The method of claim 1, wherein the method lacks the step of marrow conditioning before injection of the cells.

3. The method of claim 1, wherein the exogenous gene has been introduced into the cells using a viral vector.

4. The method of claim 1, wherein the receptor is a chimeric protein having (a) an extracellular domain of receptor 1 that dimerizes the chimeric protein in response to ligand binding, and (b) a cytoplasmic domain of receptor 2 that induces cell proliferation in response to the dimerization.

5. The method of claim 1, wherein the receptor has a cytoplasmic domain of a hematopoietic cytokine receptor.

6. The method of claim 1, wherein the receptor has a cytoplasmic domain of a thrombopoietin (TPO) receptor or a granulocyte colony-stimulating factor (G-CSF) receptor.

7. The method of claim 1, wherein the receptor has an extracellular domain of an erythropoietin (EPO) receptor.

8. The method of claim 1, wherein the cell is a pluripotent stem cell.

9. The method of claim 1, wherein the mammal is a primate.

10. The method of claim 1, wherein the method comprises the step of administrating a ligand of the receptor into the mammal.

11. The method of claim 1, wherein the cell comprises a vector having a therapeutic gene.

12. A bone marrow transplant comprising (a) lymphohematopoietic cells having an exogenous gene encoding a receptor that induces cell proliferation in response to ligand binding, and (b) a pharmaceutically acceptable carrier.

13. A kit for transplanting lymphohematopoietic cells into a mammal, wherein the kit comprises (a) a vector encoding a receptor that induces cell proliferation in response to ligand binding, and (b) a recording medium comprising a description referring to use of lymphohematopoietic cells to which the vector is introduced for injection into a bone marrow cavity.